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Single-step, single-organism bioethanol production and bioconversion of lignocellulose waste materials by phlebioid fungal species

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ABSTRACT

Ethanol production from non-pretreated lignocellulose was carried out in a consolidated bioprocess with wood-decay fungi of phlebioid Polyporales. Ethanol production was attempted on glucose, spruce wood sawdust and waste core board. Substantial quantities of ethanol were achieved, and isolate *Phlebia radiata* 0043 produced 5.9 g/L of ethanol reaching the yield of 10.4% ethanol from core board lignocellulose substrate. Acidic initial culture conditions (pH 3) induced ethanol fermentation compared to the more neutral environment. Together with bioethanol, the fungi were able to produce organic acids such as oxalate and fumarate, thus broadening their capacity and applicability as efficient organisms to be utilized for bioconversion of various lignocelluloses. In conclusion, fungi of *Phlebia* grow on, convert and saccharify solid lignocellulose waste materials without pre-treatments resulting in accumulation of ethanol and organic acids. These findings will aid in applying fungal biotechnology for production of biofuels and biocompounds.

1. Introduction

Lignocellulose is an abundant renewable resource that can be biotechnologically converted into valuable bio-products, and to ethanol by fermentation of the plant polysaccharide-derived sugars (Martínez et al., 2009; Viikari et al., 2012). Ethanol production from lignocellulose waste materials originating from forestry and agriculture is more sustainable than the first-generation bioethanol processes using food crop plants like maize as feedstock for fermentation (Martínez et al., 2009; Sarkar et al., 2012). Therefore, usage of the alternative and recycled bioprocessing source biomasses improve both the sustainability and efficiency of land use. Well-designed second-generation bioethanol production from lignocellulose thus enables the input of green energy to transportation sector (Nigam and Singh, 2011) as well as supports attainment of eco-friendly circular economy by recycling of lignocellulose-based wastes as renewable feedstock for bioprocesses and biorefineries (Liguori and Faraco, 2016; Venkata Mohan et al., 2016).

Usually, the bioconversion methods in ethanol production are preceded by strong substrate pretreatment processes such as physical and chemical methods (steam explosion, acid hydrolysis), or by enzyme treatments targeting degradation and opening up of the lignocellulose biopolymers subsequently leading to saccharification (release of dissolved sugars) (Alvira et al., 2010; Haghighi Mood et al., 2013). To produce bioethanol, pretreatments are generally followed by yeast-assisted fermentation of the released sugars, which adds an additional step and production costs into the process often referred as SSF (simultaneous saccharification and fermentation) (Antoni et al., 2007; Nigam and Singh, 2011; Olofsson et al., 2008).

These combined treatment and fermentation processes are considered efficient but they often require improvements prior to becoming economically profitable (Koppram et al., 2014; Taherzadeh and Karimi, 2008; Viikari et al., 2012). Secondly, many compounds resulting from lignocellulose pre-treatments and partial degradation (furans, phenols, and organic acids) are inhibitory for fermenting yeast cells and thereby, may lead to lower yields of bioethanol than expected (Almeida et al., 2009; Koppram et al., 2014; Viikari et al., 2012).

As an alternative, white rot Basidiomycota fungi are capable of enzymatic degradation of all the wood and lignocellulose biopolymers (Floudas et al., 2012; Kuuskeri et al., 2016; Lundell et al., 2014; Nagy et al., 2016). The fungi are capable of growing on plant biomasses directly transforming their lignocellulose substrates into fermentable sugars thus enabling so called consolidated bioprocessing (CBP) and single-step processes for ethanol production (Dionisi et al., 2015; Jouzani and Taherzadeh, 2015; Kamei et al., 2012, 2014; Sarkar et al., 2012). Biological pre-treatments and CBP have so far been of lower interest compared to chemical pre-treatment methods. One reason for this is the compatibility of CPB mainly for batch type bioreactor cultivations. Also, the recalcitrant and heterogeneous chemical structure of lignocellulose complicates control of the process, and finding optimal conditions for both saccharification and fermentation is demanding (Dionisi et al., 2015; Jouzani and Taherzadeh, 2015). Although fungal pre-treatment and CBP comprise challenges to overcome, these methods hold yet unexplored potential in bioconversion and fermentation of especially agricultural and industrial waste lignocelluloses (Liguori and Faraco, 2016; Olofsson et al., 2008; Sindhu et al., 2016).

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In this study, the CBP ability and production of bioethanol by phlebioid wood-decay white rot fungi were investigated on softwood and recycled wood-fiber based lignocellulose. The fungal isolates of current study were selected as based on their ability to grow on spruce wood while demonstrating expression of lignin-attacking and lignocellulose carbohydrate-active enzymes (Kuuskeri et al., 2015). Moreover, isolates of the genus *Phlebia* have proven versatility in bioconversion of diverse plant biomasses, and remarkable abilities in degradation and detoxification of harmful organic compounds and xenobiotics (Kamei et al., 2005; Kuuskeri et al., 2016; Lundell et al., 2014; Xiao et al., 2011). In addition to assessing bioethanol production by the fungi, analyses on carbohydrate metabolism and utilization of the solid lignocellulose substrate were conducted.

2. Materials and methods

2.1. Fungal isolates and inoculum cultivation

All fungal isolates of this study (Table 1) were selected from the HAMBI Fungal Biotechnology Culture Collection of the University of Helsinki (HAMBI-FBCC, fbcc@helsinki.fi), and their identification has been verified by ITS-PCR and sequencing (Lim et al., 2007; Floudas and Hibbett, 2015; Kuuskeri et al., 2015). Isolates of *Phlebia* were recently demonstrated to be fast at hyphal elongation and efficient in production of lignocellulose-degrading CAZy and oxidoreductase enzymes (Kuuskeri et al., 2015). For inoculation of the ethanol production and lignocellulose cultivations, the fungal isolates were pre-cultivated on 2% (w/v) malt extract 2% (w/v) agar (MEA) medium at 25 °C in the dark until the hyphal extension front reached the edges of the 8.5 cm radius petri dishes (in 5–10 days).

2.2. Culture media and substrate carbon sources

All cultivations were performed in 100 ml glass Erlenmeyer flasks filled with 20 ml of phosphate-buffered (pH 6) yeast-extract containing basal liquid medium (Okamoto et al., 2010). In another set of experiments, the medium pH was adjusted either to pH 3 or 4 by addition of hydrochloric acid. Medium was supplemented alternatively with 0.2 g (1% w/v) of glucose, 1 g of Norway spruce (*Picea abies*) sawdust wood, or 1.0–1.5 g (dry weight 5.0–7.5%) of core board lignocellulose as carbon source. Spruce wood was selected as substrate due to its importance as feedstock for board and paper industry in Finland and northern Europe (Peltola, 2014). Core board is an example of waste wood-fiber based material with no more potential for recycling or application for manufacturing. After addition of deionized water for wetting of the dry core board, the material was homogenized by milling with a sharp blade (A11 Basic analytical mill, IKA). The various carbon source supplemented growth media flasks were autoclaved at 121 °C, 15 min.

Table 1
Polyporales phlebioid fungal isolates of this study and their origins.

Code	Species	FBCC isolate number	Isolate origin	ITS sequence accession
Pace 0004	*Phlebia acerina*	0004	Birch	LN611082[a]
Pbre 1463	*Phlebia brevispora*	1463	Pine	LN611135[a]
Pbre 2371	*Phlebia brevispora*	2371	Slash pine	KP135387[b]
Pcen 0207	*Phlebia centrifuga*	0207	Spruce	LN611105[a]
Poch 0360	*Phlebia ochraceofulva*	0360	Unknown	LN611117[a]
Prad 0043	*Phlebia radiata*	0043	Alder	LN611085[a]
Prad 0125	*Phlebia radiata*	0125	Birch	LN611086[a]
Psub 0426	*Phlebia subserialis*	0426	Unknown	LN611120[a]
Ptre 0082	*Phlebia tremellosa*	0082	Birch	LN611124[a]
Pchr 0280	*Phanerochaete chrysosporium*	0280	Beech	AF475147[c]

[a] Kuuskeri et al. (2015).
[b] Floudas and Hibbett (2015).
[c] Lim et al. (2007).

2.3. Fungal cultivations for ethanol production

The fungi were inoculated by transferring a 5 mm diameter-sized fungal mycelium covered MEA plug onto the surface of the liquid medium or the solid lignocellulose substrate in the culture flasks. The flasks were sealed tightly with autoclaved rubber stoppers (top diameter 30 mm, height 35 mm, bottom diameter 29 mm). Stoppers had one inlet which was blocked with a filter-containing micropipette tip. This set-up comprised semi-aerobic culture conditions. For anaerobic culture conditions in the bioconversion optimization experiments, impermeable (intact) rubber plug stoppers (38 × 35 × 31 mm) were used. All cultivations were carried out at 25 °C, in the dark, as non-agitated flask cultures, and consisted of three flasks (three biological replicate cultures) of each fungal isolate on each carbon source studied throughout all the experiments.

Ethanol fermentation capability was investigated by cultivating the isolates in the basal medium supplemented with 1% (w/w) glucose. Samples were taken on days 0, 3, 7, 10, 14, 17, 21 and 24. Carbon metabolism experiments were then conducted on the three carbon substrates (glucose, spruce wood or core board) applied with samples taken on cultivation days 7, 14 and 21. From all of the fungal cultivations, ethanol production and sugar consumption rate were assessed along with the carbohydrate-derived metabolites. All samples were taken by carefully opening the rubber stopper of each culture flask and quickly withdrawing approximately 500 µl of the liquid phase with a sterile glass Pasteur pipette.

2.4. Dry weight (mass) analyses

In order to estimate utilization of the solid lignocellulose substrate (spruce wood or core board), combined dry weight of fungal mycelium and the remaining lignocellulose substrate were measured after 24 days of cultivation. Dry weight analysis of the substrate and mycelium was performed by drying the solids at 90 °C for 2 days after vacuum suction through glass fiber filters (GF/C, Whatman), and weighing either the combined or mycelium-detached total dry weight of each culture flask. The results are based on the mean average net dry weight values of three biological replica cultures.

2.5. Chromatographic analyses

Core board (5–10 mg dry weight portions) was hydrolyzed in 5 ml of 1 M sulfuric acid at 120 °C for 4 h, in order to determine monosaccharide composition of the lignocellulosic substrate. Hydrolysis was followed by separation and identification of the dissolved monosaccharides by HPLC-PAD analysis (Pakarinen et al., 2011). The equipment consisted of three Waters 515 HPLC pumps, 2707 autosampler, and 2465 pulsed amperometric detector. Elution was performed with a Carbopak PA1 column (4.0 mm ID × 250 mm, Dionex) by a sodium hydroxide gradient in Milli-Q water as the eluent (2–200 mM sodium hydroxide) with the flow rate of 1 ml min^{-1}. The samples were balanced to neutral

pH 7 by addition of sodium hydroxide, and filtered through 0.45 μm pore size GHP (hydrophilic polypropylene) membrane filters (Gelman) prior to injection (10 μl). Sugars were identified and quantified according to the retention time and signal intensity of reference compounds. Fucose (50 μg/ml) was used as an internal standard.

Organic acids were identified and quantified with Agilent 1290 Infinity Binary LC System (Agilent Technologies) coupled with Zorbax SB-aq RRHD column (2.1 × 15 mm, 1.8 μm particle size, Agilent Technologies). Column temperature was 40 °C, and separation was conducted at a flow rate of 0.45 min ml^{-1} under isocratic conditions by using an eluent consisting of 22.5 mM KH_2PO_4 and 17.5 mM H_3PO_4 (pH 2.37) in HPLC water. Samples were filtered through 0.2 μm pore size membrane filters (Gelman GHP) before injection (1 μl). UHPLC separation was followed at the wavelengths 210 nm, 260 nm and 280 nm, and optimized to detect carboxylic acids. Their identification was based on retention times and UV spectra of commercially available reference compounds (p.a. or p.p.a. scale purity), and quantification for oxalate and fumarate was done by using external standard reference method.

2.6. Concentration of ethanol and released sugars

Concentration of ethanol was measured by using Megazyme K-ETOH spectrophotometric-detection based assay (https://secure.megazyme.com/Ethanol-Assay-Kit) according to the instructions given by the manufacturer, and Tecan Infinite M200 microtiter plate reader. Overall concentration of dissolved reducing sugars in the cultures was determined spectrophotometrically by applying the DNS (dinitrosalicylic acid) based method (Miller, 1959).

2.7. Statistical analyses and calculations

Paired samples *t*-test was used to determine the significance ($p < 0.05$) of core board utilization applying IBM SPSS Statistics 24 software. End-product (ethanol, organic acids) concentrations were uniformly converted into moles of carbon (for details, see chapter 3.3). Theoretical maximum of ethanol production from glucose was calculated similarly as in a previous study (Kamei et al., 2014). Dry weight change after 24 days in cultures on core board was calculated by subtracting the dry weight of non-inoculated control (only core board and medium) from the dry weight of fungal-inoculated core board culture. Values from three parallel biological replica cultures and controls were used in all calculations.

3. Results and discussion

3.1. Glucose as carbon source and bioethanol production ability

Fungal isolates studied were first evaluated for their ability to utilize different carbon sources and to produce ethanol. For this, the fungi were cultivated on the glucose-supplemented medium. All isolates were able to grow on glucose (as based on production of mycelium, Appendix A Fig. S1). However, only a few fungi produced ethanol from glucose, with maximal ethanol production either on day 7, 14 or 17 (Fig. 1). The fastest producers of ethanol attained their respective production maxima within one week, and the isolates Pace 0004, Poch 0360, Prad 0043 and Pchr 0280 reached ethanol quantities of 0.8–3.45 g/L on day 7. On day 14, the highest concentrations of ethanol (0.62–2.05 g/L) were detected in cultures of the isolates Prad 0125, Psub 0426 and Ptre 0082. On day 17, low concentrations of ethanol were detected only e.g. with the isolate Pcen 0207 (0.13 g/L ethanol).

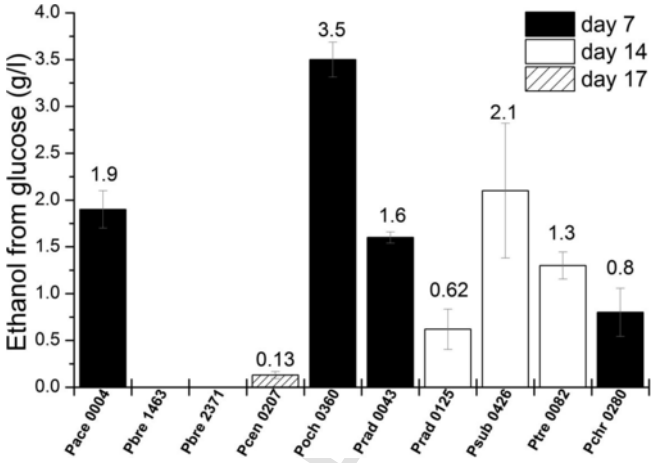

Fig. 1. Production of ethanol from glucose presenting the highest concentration per fungal isolate (either on day 7, 14 or 17 of cultivation). Mean average values of three replica cultures and their standard deviation (error bars) are shown. For fungal codes, see Table 1.

These results imply that with most of the phlebioid isolates studied, ethanol fermentation occurs within two weeks of cultivation under these conditions.

3.2. Core board as carbon source and growth substrate

All isolates were cultivated on core board in order to assess their ability to grow on and utilize the waste lignocellulose substrate. Of the fungi that were capable of fermenting ethanol from glucose, five isolates (Pace 0004, Poch 0360, Prad 0043, Prad 0125, and Pchr 0280) were also capable of utilizing the core board lignocellulose as growth substrate (Fig. 2). Isolates capable of fermenting ethanol from glucose as well as capable of degrading core board were then selected for a more thorough carbon metabolism analysis. Those fungal isolates that were both unable to produce detectable amounts of ethanol from glucose, and unable to utilize core board as growth substrate (five isolates) were thereby omitted from the following experiments.

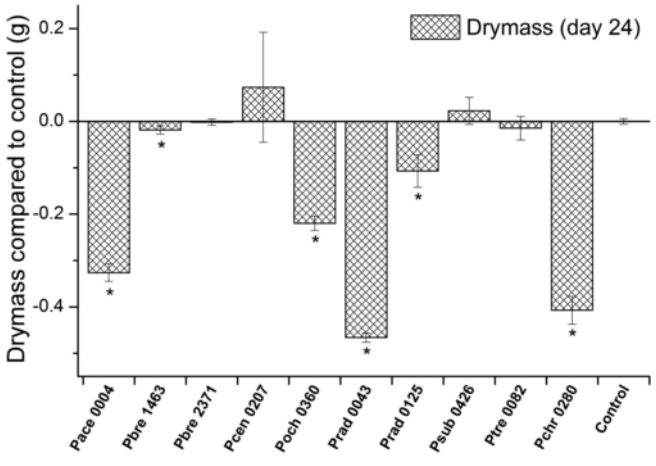

Fig. 2. Fungal ability of utilization of the core board lignocellulose substrate. Change in the dry weight of core board substrate compared to the average of non-inoculated controls after 24 days of fungal cultivation. Zero line (value 0.0) represents the dry weight of non-inoculated lignocellulose. Marked bars (by asterisk ∗) represent significant differences ($p < 0.05$). For fungal codes, see Table 1.

3.3. Carbon metabolism

Utilization of the three different organic carbon sources (glucose, spruce wood sawdust or milled core board) were followed in the fungal cultures by observing organic compounds that were secreted to the culture medium. The aim was to highlight the species-specific differences of carbon utilization and to evaluate other factors – together with ethanol production – related to bioconversion such as secretion of organic acids.

One of the most important features in bioconversion after the impairment of lignocellulose structure is how the released mono- and oligosaccharides are utilized by the organisms. Sugar consumption rate or intake efficiency (e.g. in yeast processes) is beyond dispute a key feature in biomass bioconversion or ethanol fermentation efficiency (Van Vleet and Jeffries, 2009). Regarding glucose intake by the phlebioid fungi, isolates Pace 0004, Poch 0360, Prad 0043 and Pchr 0280 were able to consume majority of the added glucose in 7 days and thus, they may be consequently considered potential for bioconversions (Fig. 3A). Isolate Prad 0125 apparently required more time for glucose consumption.

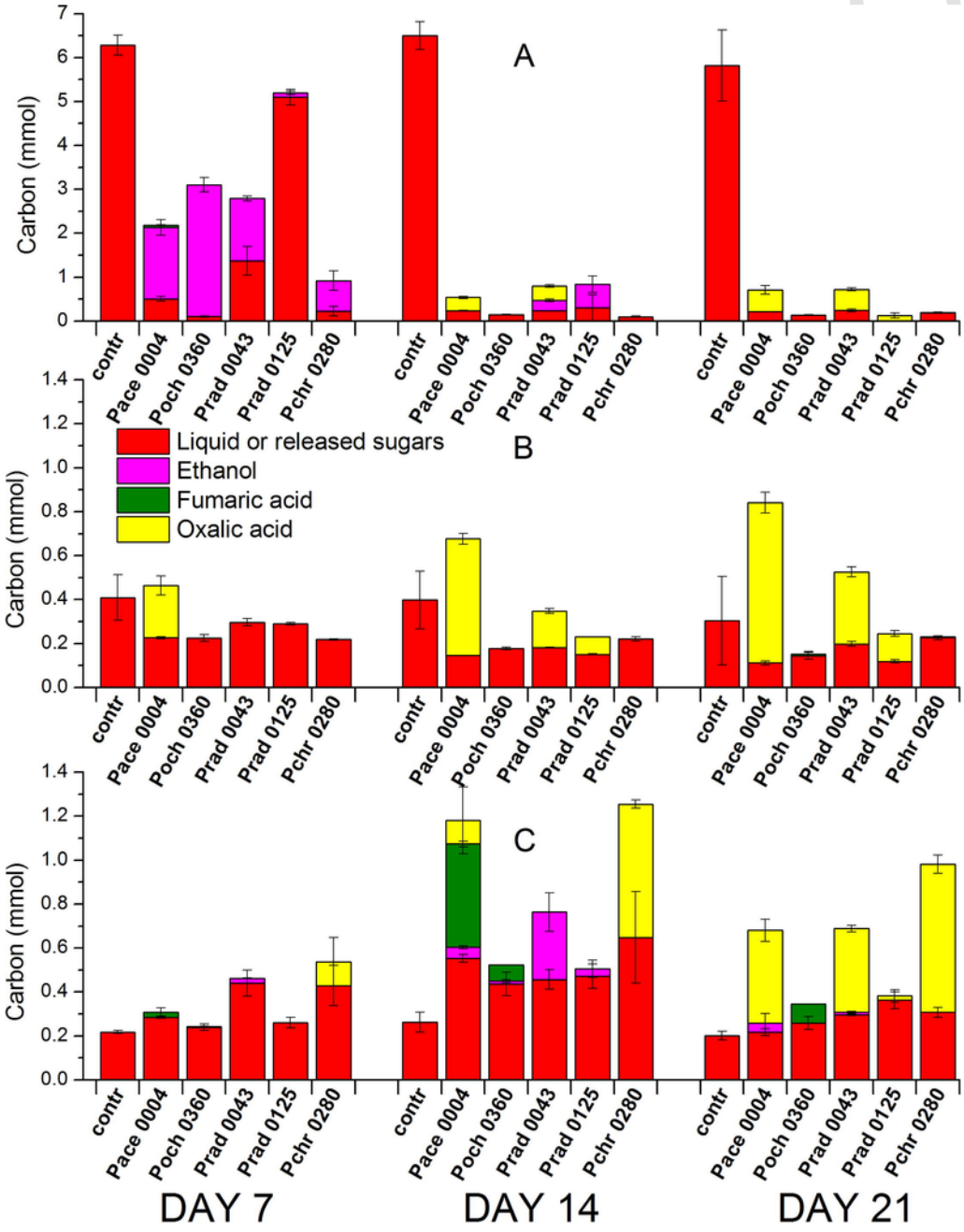

Fig. 3. Carbon metabolism of the fungal isolates at three time points (on days 7, 14 and 21) of cultivation on different carbon sources: (A) 0.2 g glucose, (B) 1 g of spruce wood, and (C) 1 g of core board. Bars with different colours represent the mean average values of molar quantities of carbon in different organic compounds, respectively, as analyzed from three parallel fungal cultures under each condition and carbon source. Error bars represent standard deviation of the three parallel flask cultures. Control values represent the non-inoculated cultivations without fungi. For fungal codes, see Table 1.

When the fungal isolates were cultivated on lignocelluloses, either on spruce wood or core board, interpretation of sugar intake efficiency was more complicated due to simultaneous decomposition of the solid lignocelluloses and consumption of the released sugars, mainly glucose. On the other hand, due to obviously slow rate of saccharification (release of sugars) from the solid lignocellulose substrates into the medium water phase, sugar intake rate and efficiency becomes less of importance regarding bioconversion and technological applications.

3.3.1. Production of oxalic acid

The predominant fungal bioproduct was oxalic acid (oxalate in dissociated form) disregarding the various carbon sources applied in the fungal cultivations (Fig. 3 A-C). The ability to produce and secrete this di-carboxylic acid is widespread among fungi in the Polyporales order, and several of the described functions of fungal-secreted oxalate are related to wood and lignocellulose decay processes (Lundell et al., 2014; Mäkelä et al., 2010). However, some preferences of the carbon source were observed. Isolates of the phlebioid species *Phlebia radiata* and *Phlebia acerina* produced oxalic acid on all three carbon sources tested, while the isolate Pchr 0280 produced oxalate only on core board substrate. Thus, it may be concluded that the core board lignocellulose contains agents which could induce fungal oxalate production. *Phanerochaete chrysosporium* is known to increase oxalate secretion at least as a response to dissolved metal ions, such as Cd^{2+} and Pb^{2+} (Li et al., 2015; Zhao et al., 2016). Thereby, fungal production of oxalate may as well be considered as a metal pollutant detoxifying method by chelation of harmful metal cations in their habitat.

Overall accumulation of oxalic acid in wood-decay fungal cultures may be a consequence of low expression or lowered activity of their oxalate-degrading enzymes such as oxalate carboxylase (ODC) (Mäkelä et al., 2010). Especially in the culture medium buffered to higher pH, ODC activities may be affected since it has been demonstrated that ODC expression and activities are induced under more acidic (lower pH) fungal culture conditions (Mäkelä et al., 2014).

3.3.2. Production of fumaric acid

Fumaric acid (fumarate in dissociated form) was initially detected in cultures of three fungal isolates, but detectable amounts were produced only by isolates Poch 0360 and Pace 0004 of which the latter produced remarkably high concentrations of fumaric acid (ca. 6 mM) equal to 0.47 mmol of carbon from 1 g of core board in each cultivation (Fig. 3C). No satisfactory biological explanation for accumulation of fumarate under these conditions may be presented. Possible hypotheses could be that fumarate is functioning as a pH mediator, or fumaric acid may be a temporal product derived from so called fumarate respiration, which may lead, however, to ATP dissipation and oxygen consumption (Shah, 2009; Taymaz-Nikerel et al., 2013). Similar production of fumarate – together with oxalate and other carboxylic acids – by a wood decaying fungus has been reported only once before (Hofrichter et al., 1999). Although the detected concentrations of fumarate were relatively low, production of fumaric acid from untreated lignocellulose (here core board) can be considered as a novel and appealing direction in lignocellulose bioconversion.

3.3.3. Bioconversion of core board to ethanol

Major focus of this study was production of bioethanol due to the social and economic importance of finding more sustainable methods for ethanol and transportation fuel production. Five isolates were able to ferment ethanol from glucose but only four of these (Pace 0004, Prad 0043, Prad 0125 and Poch 0360) were capable of producing ethanol from the waste core board lignocellulose (Fig. 3 A-C). No ethanol production was detected from spruce wood sawdust. White rot fungal initiated decomposition of lignocellulose and the degradative enzymatic reactions require oxygen (Kirk and Farrell, 1987; Lundell et al., 2014; Madhavi and Lele, 2009), and therefore, the fungal isolates were cultivated under semi-aerobic conditions which should facilitate or even increase degradation and conversion of solid lignocelluloses. Yet under semi-aerobic cultivation conditions, ethanol was quickly metabolized or degraded further which was seen as a sharp decrease in ethanol concentration at further stages of cultivation.

To overcome this issue, the most prominent *Phlebia* isolates (Pace 0004, Prad 0043 and Poch 0360) and isolate Pchr 0280 were cultivated under anaerobic conditions applying either initial pH of 6 or 3 in the cultures. Since the isolate Prad 0043 was considered as the most prominent for lignocellulose bioconversion and bioethanol production, also other conditions, such as initial pH 4 and increased proportion of the lignocellulose substrate were tested. Cultivation was shortened to 9 days since the maximal level of ethanol accumulation was repeatedly reached within this time frame (Appendix A Fig. S2).

In both cases, with initial pH 3 or 6 in the cultures, isolate Prad 0043 was the most efficient fungus reaching ethanol concentration of 5.2 g/L (Fig. 4A) and also giving the production of 0.104 g ethanol per 1.0 g of core board substrate, that is 10.4% yield (Table 2). Yet, the highest concentration of ethanol (5.9 g/L, 128 mM) was obtained with Prad 0043 on the waste lignocellulose when the dry weight percentage was increased to 7.5% (w/v) in cultures with initial acidity adjusted to pH 3 (Fig. 4A).

Lower initial culture pH adjustment was systematically observed to be more efficient for ethanol production by the phlebioid isolates (Fig. 4A–C). Lower culture pH value reflects the acidic natural growth environments of the wood-inhabiting Polyporales fungi, and may thereby promote their wood and lignocellulose decomposition processes (Lundell et al., 2014; Mäkelä et al., 2010). Also, it may be hypothesized that changes in the culture medium pH and proton concentration may affect glucose transportation and intake efficiency.

Even though biological degradation of wood and lignocellulose is an oxygen-demanding process (Kirk and Farrell, 1987), anaerobic conditions were observed in our study to be more beneficial for production of bioethanol from the waste core board lignocellulose. This is most likely explained by the impossibility for metabolizing the fermentation product ethanol in this environment (Lee, 1997). By applying acid hydrolysis combined with HPLC-PAD analysis, the waste core board was determined to contain 8.0 ± 1.8% of glucose and 8.8 ± 2.0% of xylose (of dry weight). However, these results are too low since the measured concentrations of ethanol in the core board cultivations were higher than what is theoretically possible (to ferment from this quantity of monosaccharides). For instance, manufactured card board contains cellulose up to 50–60% (Brummer et al., 2014; Wang et al., 2012). Thus, it may be theoretically estimated that the recycled core board contains around the same quantity of carbohydrates, which more supports the ethanol concentrations detected.

Yield of ethanol production by the phlebioid isolates was similar or even higher than what has been obtained with other white rot fungal isolates in pre-treatment involving SSF processes on lignocelluloses, as interpreted in equivalence to 100 g of the solid substrate used in each study (Table 2). However, most of the other processes included lignocellulose pre-treatments prior to fungal inoculation, whereas in this study, no pre-treatment methods were applied. Moreover, production of bioethanol on waste lignocellulose by phlebioid fungi is a rapid or even faster process than is reported for Basidiomycota fungi cultivated on agricultural or forest industry waste biomasses. In comparison to the SSF methods requiring lignocellulose pre-treatments, other benefits of the here presented consolidated bioprocessing method are (i) efficient and more preserving conversion of the monosaccharides released from

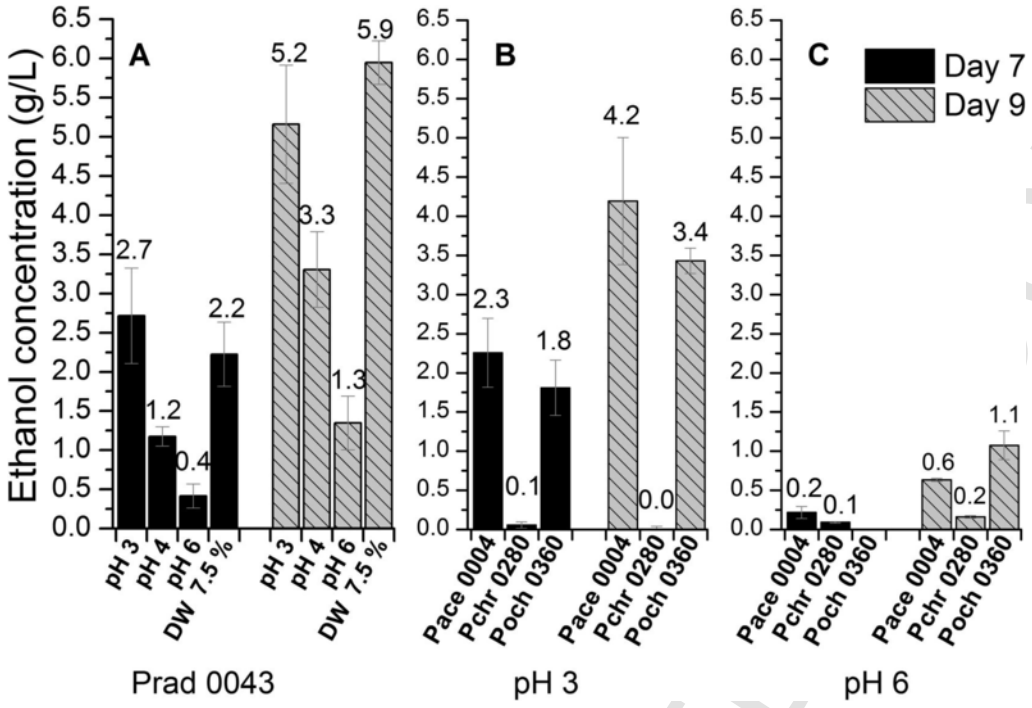

Fig. 4. Ethanol production from waste lignocellulose core board by selected fungal isolates determined on days 7 and 9 of cultivation under anaerobic conditions. (A) Isolate Prad 0043 cultivated under four different culture conditions: initial pH 3, 4, or 6 with 5.0% dry weight ratio of lignocellulose; and at pH 3, with 7.5% dry weight ratio of lignocellulose. The three other ethanol-producing phlebioid isolates were cultivated under conditions of 5.0% dry weight ratio of lignocellulose (B) at pH 3, and (C) at pH 6, respectively. Error bars represent standard deviation of three parallel culture flasks. For fungal codes, see Table 1.

Table 2
Yield of ethanol production in white rot fungal single-organism and combined organisms using fermentations on various lignocelluloses, extrapolated to 100 g of the solid substrate.

Ethanol/substrate (g/100 g)	Substrate	White rot fungus	Duration (days)	Reference
Single organism				
10.4	Core board	*Phlebia radiata* 79	9	Present study
20	Newspaper	*Phlebia* sp. MG-60	9	Kamei et al. (2012)
42	UHKP[a]	*Phlebia* sp. MG-60	7	Kamei et al. (2012)
9.9	Rice straw	*P. chrysosporium*	19	Bak et al. (2009)
Combined organisms				
1.7 (3)	Corn stover	*P. chrysosporium* (with yeast)	7 (9)	Shrestha et al. (2008)
6.6	Corn stover[b]	*P. chrysosporium*	>9	Vincent et al. (2014)
		Co-culture with a brown rot fungus and yeast		

[a] Unbleached hardwood kraft pulp.

[b] Sodium hydroxide pre-treatment.

the lignocellulose substrate (high percentage of substrate's sugars are consumed), (ii) apparent non-existence of toxic or inhibitory compounds disturbing fungal growth and fermentation, and (iii) requirement of minimum heat and power input to the process. These factors along with other requirements for a proper biotechnological conversion of lignocellulose (Alvira et al., 2010) are fulfilled in the here reported method. By further optimizing the presented fungal method for bioconversion of waste lignocellulose, it may be foreseen that even the requirements of high gravity production of lignocellulosic ethanol could be approached (Koppram et al., 2014).

4. Conclusions

This study pinpoints that single-organism driven consolidated bioconversion of lignocellulose waste is possible without pre-treatment of the solid substrate. Observed bioethanol production from waste lignocelluloses is comparable to studies including additional pre-treatments and/or several microorganisms. Fungi of the genus *Phlebia* established to be robust bioconversion organisms for production of bioethanol and organic acids, oxalate and fumarate. This feature is remarkable when considering their biotechnological potential. Production of oxalate, substrate mass loss and hydrolysis of the solid lignocellulose, and sugar release together with fungal biomass formation can also be considered as good indicators of fungal bioconversion ability on lignocellulose substrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.11.082.

References

Almeida, J.R.M., Bertilsson, M., Gorwa-Grauslund, M.F., Gorsich, S., Lidén, G., 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 82, 625–638.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. 101, 4851–4861.

Antoni, D., Zverlov, V.V., Schwarz, W.H., 2007. Biofuels from microbes. Appl. Microbiol. Biotechnol. 77, 23–35.

Bak, J.S., Ko, J.K., Choi, I.G., Park, Y.C., Seo, J.H., Kim, K.H., 2009. Fungal pretreatment of lignocellulose by Phanerochaete chrysosporium to produce ethanol from rice straw. Biotechnol. Bioeng. 104, 471–482.

Brummer, V., Jurena, T., Hlavacek, V., Omelkova, J., Bebar, L., Gabriel, P., Stehlik, P., 2014. Enzymatic hydrolysis of pretreated waste paper – source of raw material for production of liquid biofuels. Bioresour. Technol. 152, 543–547.

Dionisi, D., Anderson, J.A., Aulenta, F., McCue, A., Paton, G., 2015. The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review. J. Chem. Technol. Biotechnol. 90, 366–383.

Floudas, D., Hibbett, D., 2015. Revisiting the taxonomy of Phanerochaete (Polyporales, Basidiomycota) using a four gene dataset and extensive ITS sampling. Fung. Biol. 119, 679–719.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martínez, A.T., Otillar, R., Spatafora, J.W., Yadav, J.S., Andrea, A., Isabelle, B., Boyd, A., Carlson, A., Copeland, A., LaButti, K., Larrondo, L.F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D.J., Morgenstern, I., Morin, E., Murat, C., Nagy, L.G., Nolan, M., Ohm, R.A., Patyshakuliyeva, A., Rokas, A., Ruiz-Dueñas, F.J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J.C., St. John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D.C., Martin, F., Cullen, D., Grigoriev, I.V., Hibbett, D.S., 2012. The paleozoic origin of enzymatic ligning decomposition reconstructed from 31 fungal genomes. Science 336, 1715–1719.

Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H., Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renew. Sustain. Energy Rev. 27, 77–93.

Hofrichter, M., Vares, T., Kalsi, M., Galkin, S., Scheibner, K., Fritsche, W., Hatakka, A., 1999. Production of manganese peroxidase and organic acids and mineralization of ^{14}C-labelled lignin (^{14}C-DHP) during solid-state fermentation of wheat straw with the white rot fungus Nematoloma frowardii. Appl. Environ. Microbiol. 65, 1864–1870.

Jouzani, G.S., Taherzadeh, M.J., 2015. Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. Biofuel Res. J. 5, 152–195.

Kamei, I., Suhara, H., Kondo, R., 2005. Phylogenetical approach to isolation of white-rot fungi capable of degrading polychlorinated dibenzo-p-dioxin. Appl. Microbiol. Biotechnol. 69, 358–366.

Kamei, I., Hirota, Y., Mori, T., Hirai, H., Meguro, S., Kondo, R., 2012. Direct ethanol production from cellulosic materials by the hypersaline-tolerant white-rot fungus Phlebia sp. MG-60. Bioresour. Technol. 112, 137–142.

Kamei, I., Hirota, Y., Meguro, S., 2014. Direct fungal production of ethanol from high-solids pulps by the ethanol-fermenting white-rot fungus Phlebia sp. MG-60. Bioresources 9, 5114–5124.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. Ann. Rev. Microbiol. 41, 465–505.

Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol. 32, 46–53.

Kuuskeri, J., Mäkelä, M.R., Isotalo, J., Oksanen, I., Lundell, T., 2015. Lignocellulose-converting enzyme activity profiles correlate with molecular systematics and phylogeny grouping in the incoherent genus Phlebia (Polyporales, Basidiomycota). BMC Microbiol. 15, 217.

Kuuskeri, J., Häkkinen, M., Laine, P., Smolander, O.-P., Tamene, F., Miettinen, S., Nousiainen, P., Kemell, M., Auvinen, P., Lundell, T., 2016. Time-scale dynamics of proteome and transcriptome of the white-rot fungus Phlebia radiata: growth on spruce wood and decay effect on lignocellulose. Biotechnol. Biofuels 9, 192.

Lee, J., 1997. Biological conversion of lignocellulosic biomass to ethanol. J. Biotechnol. 56, 1–24.

Li, N., Zeng, G., Huang, D., Huang, C., Lai, C., Wei, Z., Xu, P., Zhang, C., Cheng, M., Yan, M., 2015. Response of extracellular carboxylic and thiol ligands (oxalate, thiol compounds) to Pb^{2+} stress in Phanerochaete chrysosporium. Environ. Sci. Pollut. Res. 22, 12655–12663.

Liguori, R., Faraco, V., 2016. Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. Bioresour. Technol. 215, 13–20.

Lim, Y., Baik, K., Chun, J., Lee, K.H., Jung, W.J., Bae, K.S., 2007. Accurate delimitation of Phanerochaete chrysosporium and Phanerochaete sordida by specific PCR primers and cultural approach. J. Microbiol. Biotechnol. 17, 468–473.

Lundell, T.K., Mäkelä, M.R., de Vries, R.P., Hildén, K.S., 2014. Genomics, lifestyles and future prospects of wood-decay and litter-decomposing Basidiomycota. In: Martin, F. (Ed.), The Fungi. Adv. Bot. Res.. vol. 70, pp. 329–370.

Madhavi, V., Lele, S.S., 2009. Laccase: properties and applications. BioResources 4, 1694–1717.

Mäkelä, M.R., Hildén, K., Lundell, T.K., 2010. Oxalate decarboxylase: biotechnological update and prevalence of the enzyme in filamentous fungi. Appl. Microbiol. Biotechnol. 87, 801–814.

Mäkelä, M.R., Sietiö, O.-M., de Vries, R.P., Timonen, S., Hildén, K., 2014. Oxalate-metabolising genes of the white-rot fungus Dichomitus squalens are differentially induced on wood and at high proton concentration. PLoS ONE 9, e87959.

Martínez, A.T., Ruiz-Dueñas, F.J., Martínez, M.J., del Río, J.C., Gutiérrez, A., 2009. Enzymatic delignification of plant cell wall: from nature to mill. Curr. Opin. Biotechnol. 20, 348–357.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Nagy, L.G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., Sun, H., Yadav, J.S., Pangilinan, J., Larsson, K.-H., Matsuura, K., Barry, K., LaButti, K., Kuo, R., Ohm, R.A., Bhattacharya, S.S., Shirouzu, T., Yoshinaga, Y., Martin, F.M., Grigoriev, I.V., Hibbett, D.S., 2016. Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. Mol. Biol. Evol. 33, 959–970.

Nigam, P.S., Singh, A., 2011. Production of liquid biofuels from renewable resources. Prog. Energy Combust. Sci. 37, 52–68.

Okamoto, K., Imashiro, K., Akizawa, Y., Onimura, A., Yoneda, M., Nitta, Y., Maekawa, N., Yanase, H., 2010. Production of ethanol by the white-rot basidiomycetes Peniophora cinerea and Trametes suaveolens. Biotechnol. Lett. 32, 909–913.

Olofsson, K., Bertilsson, M., Lidén, G., 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 1, 1–14.

Pakarinen, A., Maijala, P., Jaakkola, S., Stoddard, F.L., Kymäläinen, M., Viikari, L., 2011. Evaluation of preservation methods for improving biogas production and enzymatic conversion yields of annual crops. Biotechnol. Biofuels 4, 20.

Peltola, A., 2014. Finnish Statistical Yearbook of Forestry. Official Statistics of Finland. Agriculture, Forestry and Fishery. Finnish Forest Research Institute, Metsäntutkimuslaitos, Vantaa, Finland.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. Renewable Energy 37, 19–27.

Shah, M.V., 2009. Transport and metabolism of fumaric acid in Saccharomyces cerevisiae in aerobic glucose-limited chemostat culture. Yeast 26, 545–551.

Shrestha, P., Rasmussen, M., Khanal, S.K., Pometto, A.L., Van Leeuwen, J., 2008. Solid-substrate fermentation of corn fiber by Phanerochaete chrysosporium and subsequent fermentation of hydrolysate into ethanol. J. Agric. Food Chem. 56, 3918–3924.

Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass – an overview. Bioresour. Technol. 199, 76–82.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int. J. Mol. Sci. 9, 1621–1651.

Taymaz-Nikerel, H., Jamalzadeh, E., Borujeni, A.E., Verheijen, P.J.T., van Gulik, W.M., Heijnen, S.J., 2013. A thermodynamic analysis of dicarboxylic acid production in microorganisms. In: von Stockar, U., van der Wielen, L.A.M. (Eds.), Biothermodynamics: The Role of Thermodynamics in Biochemical Engineering pp. 547–579.

Van Vleet, J.H., Jeffries, T.W., 2009. Yeast metabolic engineering for hemicellulosic ethanol production. Curr. Opin. Biotechnol. 20, 300–306.

Venkata Mohan, S., Nikhil, G.N., Chiranjeevi, P., Nagendranatha Reddy, C., Rohit, M.V., Kumar, A.N., Sarkar, O., 2016. Waste biorefinery models towards sustainable circular bioeconomy: critical review and future perspectives. Bioresour. Technol. 215, 2–12.

Viikari, L., Vehmaanperä, J., Koivula, A., 2012. Lignocellulosic ethanol: from science to industry. Biomass Bioenergy 46, 13–24.

Vincent, M., Pometto, A.L., van Leeuwen, J.H., 2014. Ethanol production via simultaneous saccharification and fermentation of sodium hydroxide treated corn stover using Phanerochaete chrysosporium and Gloeophyllum trabeum. Bioresour. Technol. 158, 1–6.

Wang, L., Templer, R., Murphy, R.J., 2012. High-solids loading enzymatic hydrolysis of waste papers for biofuel production. Appl. Energy 99, 23–31.

Xiao, P., Mori, T., Kamei, I., Kiyota, H., Takagi, K., Kondo, R., 2011. Novel metabolic pathways of organochlorine pesticides dieldrin and aldrin by the white rot fungi of the genus Phlebia. Chemosphere 85, 218–224.

Zhao, M., Zhang, C., Zeng, G., Cheng, M., Liu, Y., 2016. A combined biological removal of Cd^{2+} from aqueous solutions using Phanerochaete chrysosporium and rice straw. Ecotoxicol. Environ. Saf. 130, 87–92.